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SHORT COMMUNICATION

Identification of a Neutralizing Epitope on an 82-kDa Protein Encoded by Gene 3 of Group C Rotavirus

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A neutralizing monoclonal antibody (1A5) with hemagglutination inhibition activity was produced against a cultivable porcine group C rotavirus strain (strain AmC-1) and used to identify and map the functional topography of the protein involved in viral neutralization. The 1A5 mAb recognized an 82-kDa protein from infected cells as well as an equivalent *in vitro*-translated protein programmed with gene 3 of the AmC-1 strain. By employing a method called DNA amplification-restricted transcription–translation a series of C-terminal-truncated fragments of the 82-kDa protein was produced. Immunoprecipitation experiments show that 1A5 recognizes an epitope located on vp5, a tentative trypsin cleavage fragment of the 82-kDa protein. The functional and antigenic topography on the 82-kDa protein is discussed in relation to vp4 of group A rotavirus.

Until recently it was believed that all rotavirus strains, irrespective of the species from which they were isolated, shared a common group antigen. During the last decade, however, reports on morphologically identical but antigenically distinct rotaviruses have been published (*1–7*). Based upon RNA profile and immunological characteristics they were assigned to groups B–E (*8*). Serious difficulties in cultivating nongroup A rotaviruses have limited the amount of information available concerning their molecular and biological relation to group A rotaviruses. No information is yet available about which proteins are involved in protective immunity or which epitopes induce neutralizing antibodies. Also, the complete gene coding assignment for group C rotavirus has not yet been established.

In this study we report the production and characterization of the first nongroup A rotavirus monoclonal antibody with neutralizing capacity. We also show that gene 3 of the porcine group C AmC-1 strain encodes an 82-kDa protein and that the protein under native conditions is capable of inducing not only neutralizing antibodies but also antibodies with hemagglutination inhibition capacity. A DNA amplification-restricted transcription–translation (DARTT) assay, previously used to map vp4 epitopes of group A rotavirus (*9*), was employed to topographically map the domain mediating neutralization. The neutralizing monoclonal antibody (mAb) recognized an epitope within amino acids 283–468 located to a tentatively designated vp5* trypsin cleavage fragment of vp4 (*10*).

Group C rotavirus mAbs against a cultivable porcine group C rotavirus (strain AmC-1) were produced by intra-

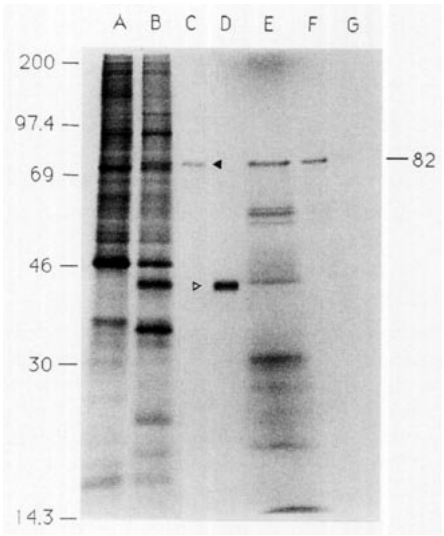

FIG. 1. Identification of *in vivo*- and *in vitro*-translated vp4. Polypeptide separation was performed by SDS–PAGE using 10% separation gel. Lane A, Mock-infected swine testicular (ST) cells labeled with [^{35}S]methionine. Lane B, Group C rotavirus-infected cells labeled with 50 mCi [^{35}S]methionine. Lane C, Immunoprecipitation of native 82-kDa protein from lysate in lane B with mAb 1A5. Lane D, Immunoprecipitation of lysate in lane B with anti-vp6 group C mAb (8G5). Lane E, *In vitro* translation of gene 3 from pGEM-3Z vector. Lane F, Immunoprecipitation of protein from lane E with mAb 1A5. Lane G, Immunoprecipitation of lysate in lane E with mAb 8G5 (vp6). Molecular weight markers are shown on the left.

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TABLE 1

Oligonucleotide Primers Used to Identify a Neutralizing Domain on the 82-kDa Protein Encoded by Gene 3 of the AmC-1 Strain of Group C Rotavirus

Primer	Position	Sequence
CG3-3′A	611–630	5′-CATCACTGCGTGGAAATTCA-3′
CG3-3′B	851–870	5′-CACCTTTGACTACTTCTGAT-3′
CG3-3′C	1391–1410	5′-CTGTTTGAGAAGTATGCCCG-3′
CG3-3′D	1801–1820	5′-AGTTGATACCATCACAGCGG-3′
CG3-3′E	2196–2216	5′-AAGCTTGGATCCCTATAATCTGCATTGTCTTAA-3′ (*Bam*HI)
CG3-T3-5′	6–23	5′-CCCGAATTAACCCTCACTAAAGATGGCGTCCTCACTTTAT-3′ (T3)
CG3-5′	6–23	5′-GAATTCGGATCCATGGCGTCCTCACTTTAT-3′ (*Bam*HI)
CG3-717-737	717–737	5′-TTTCATCTCAAAAGTTGTAAT-3
CG3-729-749	729–749	5′-TATTTTCATCATTTTCATCTC-3

peritoneal immunization of 7- to 8-week-old female Balb/c mice with purified double-shelled virus in complete Freund's adjuvant. Porcine group C rotavirus was propagated with trypsin (1 μg/ml) in swine testicular (ST) cells and purified as described (*11–13*). After two additional boosts in incomplete Freund's adjuvant, mice were given an intravenous boost in PBS. Two days after the final boost, spleen cells were fused with sp2/0 myeloma cells. Culture supernatants containing group C-specific antibodies were identified by immunoperoxidase staining on group C rotavirus-infected ST cells and cloned three times by limiting dilution. Immunoglobulin isotype was determined by Ouchtherlony immunodiffusion. For ascites production, pristane-primed Balb/c mice were injected intraperitoneally with cloned hybridoma cells. A previously established fluorescence focus reduction test for group C rotavirus (*11*) was employed to screen mAbs for neutralizing capacity. Briefly, equal volumes of mAbs in twofold serial dilutions and 500 focus-forming units of group C rotavirus were mixed and incubated for 1 hr at 37°, followed by inoculation of confluent ST cells in eight-well Lab-Tek chambers. After 1 hr of incubation at 37°, cells were washed twice with Eagle's MEM, and infection was allowed to continue for 16 hr at 37° before fixation in acetone and staining of infected cells by immunofluorescence (*11*). Neutralization titers were expressed as the reciprocal of the highest dilution giving a 60% reduction in the number of infected cells compared to a virus control. A mAb designated 1A5 (isotype IgG1) with a neutralizing titer of 5200 was selected for further characterization. The recent observation that AmC-1 strain possesses a hemagglutinin (*11*) stimulated us to establish a hemagglutination inhibition (HI) test to examine if 1A5 had HI activity. The HI assay was performed essentially as described (*11*), by mixing 25 μl of mAb (ascites) in twofold serial dilutions with 25 μl of 4 HA units of virus in PBS containing 0.5% BSA. After 1 hr at room temperature, 50 μl of 0.5% fresh human type 0 erythrocytes in PBS, 0.5% BSA was added. The inhibition titer was expressed as the minimum dilution inhibiting 4 HA units. Mab 1A5 (ascites) was found to have a HI titer of 256 in contrast to a group C anti-vp6 mAb (8G5) which showed no HI activity (<4).

To establish the protein specificity of mAb 1A5, a radioimmunoprecipitation assay (RIPA) was established as described (*13, 14*). Briefly, ST cells were infected with trypsin-activated group C rotavirus (10 PFU/cell) and metabolically labeled for 1 hr with 50 mCi [^{35}S]methionine at 7 hr p.i. As seen in Fig. 1, mAb 1A5 recognized an 82-kDa protein from infected cells. Gene 3 of the group C Cowden strain has recently been sequenced and predicted to code for an 83-kDa protein with significant sequence homology, including putative trypsin cleavage sites, to vp4 of various group A isolates (*10*). As the MW of the protein from infected cells recognized by 1A5 was similar to that of the deduced protein product of gene 3, we wanted to determine if the neutralizing antibody actually recognized the gene 3 product of the AmC-1 strain. Briefly, group C rotavirus dsRNA was phenol–chloroform extracted from infected cells and purified on silicon dioxide (Sigma) before denaturation with methyl mercuric hydroxide. A full-length cDNA clone corresponding to RNA segment 3 was synthesized by reverse transcriptase followed by polymerase chain reaction (PCR) with primers selected (Table 1) from the 5′ (CG3-5′) and 3′ ends (CG3-3′E) of gene 3 (*10*). PCR was performed by denaturation at 94° for 1 min, annealing at 45° for 2 min, and polymerization for 2 min at 72° by *Taq* polymerase (Perkin–Elmer) for 25 cycles. The reaction was completed at 72° for 7 min. After electrophoresis in 1% agarose and gel purification, the full-length 2210-bp product was *Bam*HI-digested and ligated with T4 DNA ligase into a dephosphorylated *Bam*HI-digested pGEM-3Z vector (Promega). *Escherichia coli* JM109 was transformed with ligation products by standard procedures. Recombinants in T7 RNA polymerase promotor orientation were selected and used for

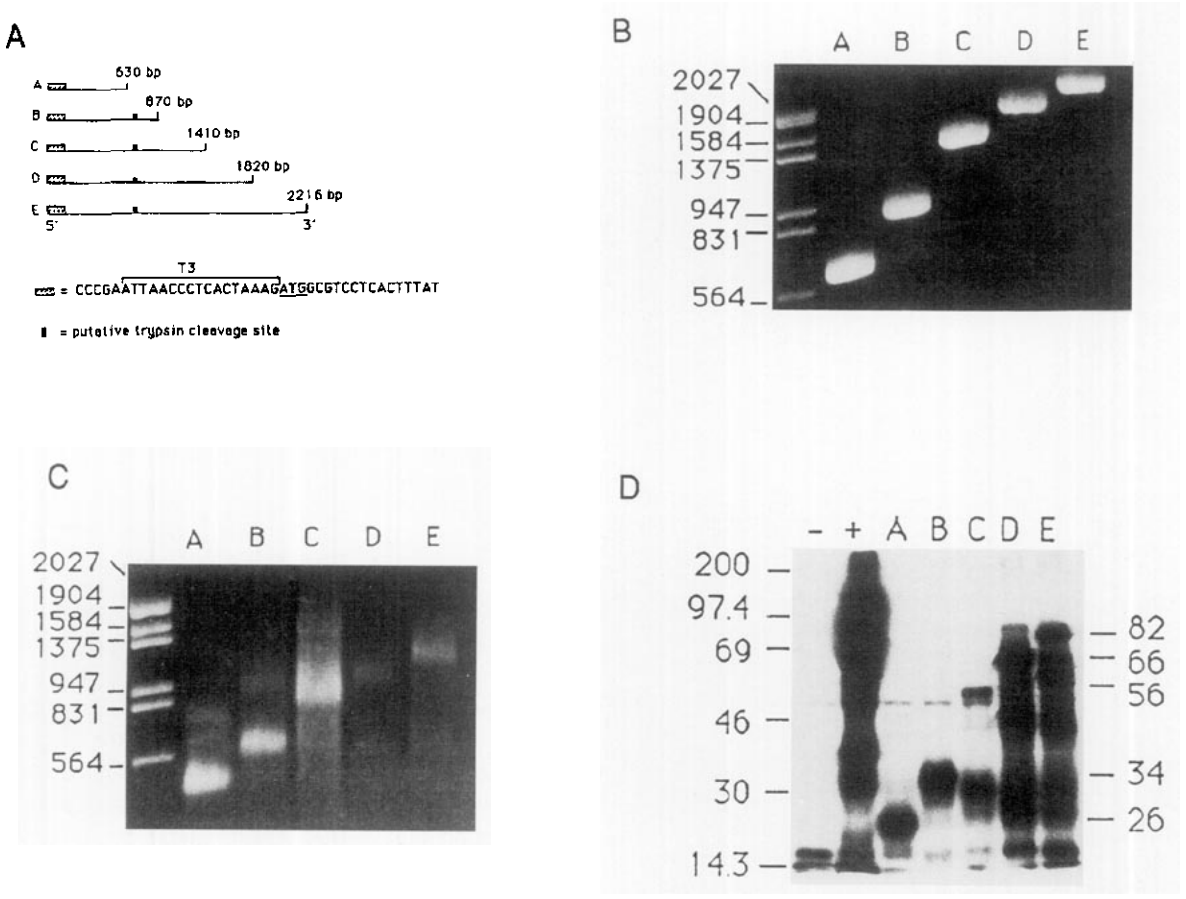

FIG. 2. Schematic presentation of 3′-truncated PCR products, PCR-amplified DNA, mRNA, and *in vitro*-translated proteins generated from gene 3 of the AmC-1 strain. (A) Stippled rectangles represent 5′-terminal sequence showing the T3 transcription initiation signal sequence. Native translation initiation signal is underlined. Putative trypsin cleavage site is marked as a black bar. (B) Presentation of native and 3′-truncated PCR amplimers after electrophoresis in a 1% agarose gel and ethidium bromide staining. DNA markers are shown on the left. (C) T3-transcribed mRNA from DNA in B. (D) Proteins translated in a rabbit reticulocyte lysate programmed with mRNA from C. Proteins were separated by SDS–PAGE (10%) under reducing conditions. Negative and positive (brome mosaic virus mRNA) translation controls are shown on the left. Molecular weight markers are shown on far left. Calculated molecular weights of native and truncated proteins are shown on the right.

transcription according to the manufacturer's instructions (Promega). Run-off mRNA transcripts were phenol–chloroform extracted and EtOH precipitated before *in vitro* translation in a rabbit reticulocyte lysate (RRL) (Boehringer Mannheim). Briefly, 500 ng of mRNA was added to 10 μl of RRL with 10 μCi [^{35}S]methionine (Amersham) and 4 units RNasin (Promega). After 1 hr incubation at 30°, the translation cocktail was diluted in 100 μl RIPA buffer (*14*) and examined by SDS–PAGE. As seen in Fig. 1, lane E, gene 3 produced a protein with a MW identical to that of the protein immunoprecipitated by 1A5 from infected cells. Furthermore, the *in vitro*-translated protein was also recognized by mAb 1A5 (Fig. 1, lane F) but not by the group C vp6 mAb 8G5 (Fig. 1, lane G), which suggests that gene 3 of porcine (AmC-1 strain) group C rotavirus encodes an 82-kDa protein capable of inducing a neutralizing antibody response.

The results stimulated us to map the antibody binding region on the 82-kDa protein. We chose to employ an elegant procedure (DARTT) developed by Mackow *et al.* (*9*) to map antibody binding sites of vp4 from group A rotavirus. DARTT is used to produce truncated or nested polypeptides using PCR with 5′ primers containing T3 RNA polymerase transcription initiation signals. To generate a defined set of C-terminal-truncated protein fragments, PCR was applied to the full-length gene 3 cDNA inserted into the pGEM-3Z vector. As shown in Fig. 2 and Table 1, the 5′ primer (CG3-T3-5′) contained an 18-base stretch of the gene 3 sequence including the native ATG translational start signal and an upstream T3 transcription initiation signal. Four different 3′ primers were used to generate truncations in the C-terminus (Table 1). After 25 cycles of PCR amplification, the PCR products were transcribed by T3 RNA polymerase and resulting mRNA was translated in RRL (Figs. 2B–2D) as described (*9*). Briefly, PCR products were phenol–chloroform extracted and EtOH precipitated. One microgram of DNA was added to a transcription mixture containing 40 m*M* Tris–HCl, 8 m*M* $MgCl_2$, 2 m*M* spermidine, 25 m*M* NaCl, 0.4 m*M* rNTP, 15 m*M* DTT, 4 U RNasin (Promega), and 100

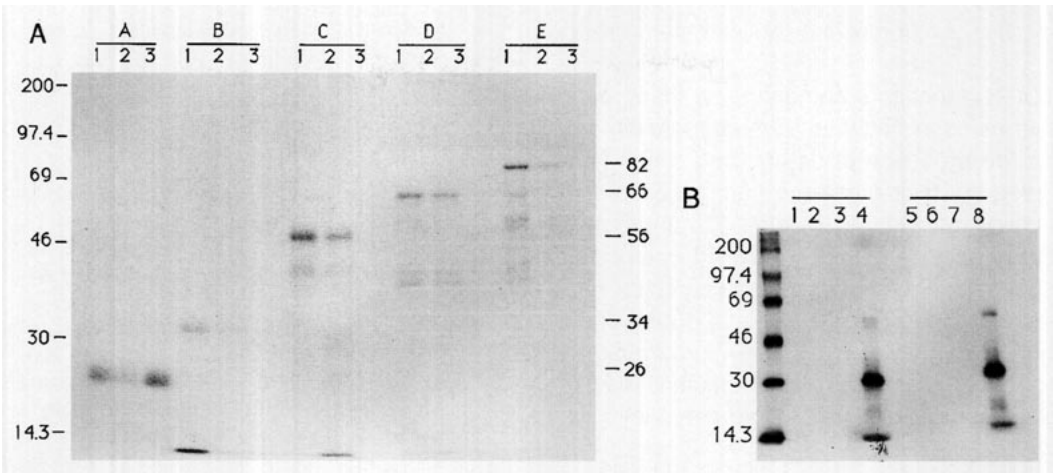

FIG. 3. Radioimmunoprecipitation of native and truncated vp4 from Fig. 2D. Lanes A to E represent truncated and native polypeptides of vp4 immunoprecipitated with a polyclonal (mouse) anti-group C antiserum (lane 1, A–E), mAb 1A5 (lane 2, A–E), and an anti-group A vp4 (M11) mAb (lane 3, A–E). Molecular weight markers are shown on the left and estimated MWs (kDa) of truncated polypeptides are shown on the right. (B) Radioimmunoprecipitation of two further carboxyl-truncated polypeptides, in size located between lanes A and B (A) and covering the trypsin cleavage sites. The truncated proteins were produced as in Figs. 1 and 2. The first lane shows immunoprecipitation of a truncated protein (aa 1–244) produced by primer CG3-T3-5′ and 3′ primer CG3-717-737 (Table 1). Lane 1, Polyclonal (mouse) anti-group C antiserum. Lane 2, mAb 1A5. Lane 3, mAb M11. Lane 4, *In vitro* translation. Lanes 5–7 show immunoprecipitation of a truncated protein spanning aa 1–248 and include the complete vp8 trypsin fragment of vp4. The truncated protein was produced by primers CG3-T3-5′ and CG3-729-749 (Table 1). Lane 5, Polyclonal (mouse) anti-group C antiserum. Lane 6, mAb 1A5. Lane 7, mAb M11. Lane 8, *In vitro* translation. Molecular weight markers are shown on the left.

U of T3 RNA polymerase (Promega) and incubated for 1 hr at 37°. Run-off transcripts were phenol–chloroform extracted and EtOH precipitated before *in vitro* translation. As seen in Fig. 2B and 2C, four sets of 3′-truncated DNA and corresponding mRNA were generated. The truncated polypeptides, ranging in MW from 26 to 66 kDa (Fig. 2D), were used in a RIPA to identify the binding domain of mAb 1A5. Both a polyclonal mouse anti-group C rotavirus antiserum and the neutralizing mAb (1A5) were able to immunoprecipitate the full-length protein and the two largest C-terminus-truncated polypeptides, whereas the second smallest protein fragment was recognized by the polyclonal rabbit antiserum but not by mAb 1A5. An irrelevant group A vp4 mAb (M 11, supplied by H. Greenberg) did not recognize either of these protein fragments. The smallest truncated polypeptide was, however, immunoprecipitated by the polyclonal rabbit antiserum, the group C mAb 1A5, and the irrelevant group A mAb (Fig. 3A), suggesting this fragment was aberrantly folded, leading to nonspecific binding.

To gain information whether the polyclonal antiserum was able to recognize a complete vp8 peptide, two carboxyl-terminal deletions including the putative trypsin cleavage sites were created. The polypeptides were created with 3′ primers CG3-717-737 and CG3 729-749 (Table 1). As seen in Fig. 3B, none of the antisera examined recognized the vp8 trypsin fragment, suggesting that vp8 of group C rotavirus has low immunogenicity or, more likely, that the virus particles used for immunizations lack the vp8 trypsin fragment.

The presented data show that one (1A5) of six mAbs (Table 2) has neutralizing capacity and that this mAb recognizes an epitope located on vp5, the tentative trypsin cleavage fragment of the 82-kDa protein (*10*). The epitope is located in the amino-terminus of the putative vp5* trypsin cleavage fragment. It is interesting to compare this observation with the proposed binding sites reported for homotypic and heterotypic neutralizing mAbs of group A rotavirus. The accumulated data concerning vp4 of group A rotavirus indicate that neutralizing mAbs, mapping to the regions on vp8, are almost exclusively serotype specific, whereas antibodies recognizing the vp5 fragment are mostly serotype crossreactive (*15–18*). The lack of cultivable group C rotavirus strains other than the Cowden and AmC-1 strains (identical serotypes) (*12, 19*) and lack of previous antigenic topography information on group C vp4 make it difficult to speculate

TABLE 2

Monoclonal Antibodies Directed Against the AmC-1 Porcine Group C Rotavirus

Name	Subclass	Protein[a]	IH[b]	NT[c]	HI[d]
1A5	IgG1	vp4	+	+	+
4B6	IgM	ND[e]	+	–	–
C2A9	NT	vp2	+	–	–
8G5	IgG1	vp6	+	–	–
8G6	IgG1	vp6	+	–	–
20G3	ND	ND	+	–	–

[a] Protein specificity. Determined by radioimmunoprecipitation assay.
[b] Immunohistochemistry. Immune peroxidase staining.
[c] Neutralization test.
[d] Hemagglutination inhibition test.
[e] ND, Not determined.

whether mAb 1A5 is homotypic or serotype cross-reactive.

Most, but not all, functional and antigenic topography of group A vp4 has been obtained from sequencing mutants grown in the presence of neutralizing mAbs (*16–18, 20*). However, the lack of plaque purification procedures for selection of escape mutants of nongroup A rotaviruses leads us to employ the DARTT method to conduct epitope mapping. As no previous search for neutralizing domains or topography analysis of outer capsid proteins of group C rotavirus has been reported, the DARTT method offered a simple and efficient way to recognize not only linear but also conformationally dependent epitopes.

Previous studies have shown that the hemagglutinin domain of group A rotavirus is located in the vp8 fragment (*21*) and that mAbs mapping not only to vp8 but also to vp5 fragments inhibit hemagglutination (*22*). With this information and the presented data, it is not yet realistic to speculate about the location of the group C hemagglutinin domain.

At present the functional and antigenic topography of vp4 from group A and C rotaviruses includes a presence of a hemagglutinin, requirement (animal strains) of sialic acid for erythrocyte and cell receptor binding (*11*), and capacity of vp4 to induce neutralizing antibodies. Other conservations between group A and C rotaviruses include the unique maturation and retention of the virus in the ER (*23*) and an icosahedral symmetry of $T = 13$ (unpublished data).

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